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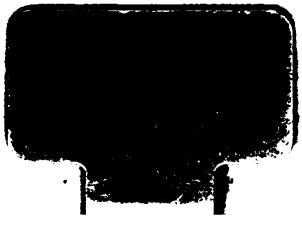

A Chemical Study of Yellow Elastic Connective Tissue.

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A CHEMICAL STUDY OF YELLOW ELASTIC CONNECTIVE TISSUE.

I. INTRODUCTION.

In order to comprehend the physiological function of any tissue, the relation which it bears to any other or to the organism of which it forms a part, and to recognize the changes which are associated with its life and growth, accurate information concerning its chemical composition is necessary. The group of connective tissues forms a class the members of which are related both as regards their mode of origin and their constituent elements. They resemble each other also in that the physical character of each is dependent on the presence in largely predominating amount of one particular constituent, usually albuminoid in nature. Until recently these tissues have been studied principally with reference to this preponderating component. Deductions regarding many of the other substances which might be associated with it have been made from inference rather than observation.

Less than any of the class has the yellow elastic tissue been studied except with reference to its main constituent, elastin. Concerning other proteid substances or those which might arise from the metabolism of proteid only the most indefinite statements are to be found. A few preliminary tests, however, have served to show us that the amount of simple (coagulable) proteid and mucoid obtainable from the ligamentum nuchae of the ox was more than would be expected from an extravascular tissue having a purely mechanical function.

With these considerations in mind we have thought it a matter of importance to subject this form of connective tissue to closer study in the hope that information gained regarding substances not already specifically investigated might furnish a rational basis for the comparison of this with other tissues, and might lead to the development of more logical methods of research into those constituents of which much is already known; and further, that a

combination of such details might throw more light on the general metabolism of the tissue in question.

In all of our investigations the source of the yellow elastic tissue has been the ligamentum nuchae of the ox.

II. COAGULABLE PROTEIDS.

At the beginning of this work we were surprised by the large amount of coagulable proteid which could be separated from the fresh ligament. If the water or saline extracts of the tissue are boiled after the addition of a trace of acetic acid, an abundant flocculent coagulum is obtained, having all the characteristics of coagulated proteid. In two determinations of the amount which could be obtained from the cleaned tissue by extraction with water we found that 0.64 per cent. of the fresh tissue or 1.93 per cent. of the dry tissue existed in this form.[50]

In order to determine, if possible, the number of these proteids we have made use of the method of fractional heat coagulation. Several extracts of the tissue were made, both aqueous and saline. 5 per cent. magnesium sulphate was most generally used for the latter type.

The method of extracting the tissue was as follows. Only such portions of the ligament as appeared free from blood were used. After a thorough cleaning of all extraneous matter—fat and connective tissue membranes—the ligaments were cut into narrow longitudinal strips and washed in cold running water for from twelve to twenty-four hours. They were then partially dried with a towel, and run through a meat chopper several times. The finely minced substance was then treated with enough fluid to cover it. At the end of twelve to twenty-four hours the fluid was strained through cloth and filtered through paper. Spectroscopic examination showed the absence of haemoglobin in every extract—an observation which implies the absence of most of the lymph proteids as well. Each fluid thus obtained became very turbid on heating, and on the addition of a drop of dilute acetic acid a flocculent coagulum separated.

In determining the temperatures of coagulation the process described by Gamgee[16] was employed—from 20-40 cc. of the extract, made faintly acid with acetic acid, being taken for each series of

determinations. The temperature was raised very gradually, never more than one degree in two minutes. When turbidity appeared the source of heat was immediately removed and the temperature held constant or raised very slowly until the turbidity had developed into a flocculent coagulum. When the separation had become distinct, usually after about half an hour had elapsed, the fluid was filtered. The filtrate in each case was as clear as water. The filtered fluid was then subjected to further heating until turbidity again ensued. This process was repeated until no more proteid could be separated from the solution. In almost every instance, an interval of several degrees occurred between the temperature at which the coagulum separated in flocculent form and the temperature at which the next succeeding turbidity appeared. Working in this way we have obtained separations at the following temperatures:

No.	Extremes of temperature.*	Average temperature.
(1)	31°-49° C.	40° C.
(2)	51°-61° C.	56° C.
(3)	60°-70° C.	65° C.
(4)	74°-76° C.	75° C.
(5)	77°-85° C.	82° C.

All of these substances were obtained from each of the types of extract used, both aqueous and saline. Nos. (4) and (5) were found in the smallest amounts.

The question naturally arose as to whether these separations represented individual proteids in the tissue. The solution of this problem we have sought in fractional separation by means of neutral salts in the methods used by Halliburton,[17] Hofmeister,[22] Kauder[25] and others.

Our results on extracts of the ligament may be summarized briefly as follows:

A. Aqueous extracts treated with $(NH_4)_2SO_4$ in substance.

(*a*). When the aqueous extract was half saturated with $(NH_4)_2SO_4$ a fairly heavy precipitate was obtained which consisted, theoretically, for the most part of globulins, albumin not being precipitated by this proportion of $(NH_4)_2SO_4$. The $MgSO_4$ solution of this substance when tested by fractional heat coagulation was found to contain bodies (1), (2), and (4) in the table

* These extremes represent the limits of all our observations. As a rule, the separations occurred at or about the mean temperature, with comparatively long intervals.

above. When the same precipitate was dissolved in water, the solution contained bodies (1), (3), (4), and (5).

(*b*). The filtrate from the precipitate obtained in (*a*) was saturated with $(NH_4)_2SO_4$. The substance so obtained was dissolved in water, the solution heated, and found to yield bodies (2), (3), (4), and (5).

B. *$MgSO_4$ extracts treated with $MgSO_4$ in substance.*

(*a*). When the $MgSO_4$ extract was saturated with $MgSO_4$ a heavy precipitate was obtained, which when dissolved in 5 per cent. $MgSO_4$ solution and heated yielded separations corresponding to (1) and (2),

(*b*). The filtrate from precipitate (*a*) on heating was found to contain bodies (2), (3), (4), and (5).

A comparison of these figures will show that of the total number of bodies present in the aqueous and saline extracts of the ligament, only one was separated by saturation with magnesium sulphate or by half saturation with ammonium sulphate, *viz*: No. (1), which on heating of the extracts separates at about 40° C. All of the other substances are to be found in the precipitates formed by saturation with magnesium sulphate or by half saturation with ammonium sulphate as well as in the filtrates from these precipitates.

C. *Fractional precipitation of aqueous and $MgSO_4$ extracts with $MgSO_4$ and $(NH_4)_2SO_4$ in substance and with saturated solution of $(NH_4)_2SO_4$.*

A closer differentiation of these proteids was desirable. To this end we have applied the following method. To a measured portion of the carefully neutralized fluid was added the precipitating salt, a few grams at a time. As soon as enough had been added to bring about a flocculent separation, the precipitate was filtered off and washed with a solution of the precipitating salt equivalent in strength to that of the mother liquid. To the filtrate plus enough of the washings to make it up to the volume of the fluid before filtration was again added weighed quantities of the precipitating salt. When a second precipitate appeared it was filtered off, washed, and the filtrate treated in a manner similar to that just described. This process was continued until all the proteid was removed or until the fluid was saturated. Each precipitate thus obtained was dissolved in a small amount of water with the aid of the salt mechanically adhering to it and the solution subjected to the process of heat coagulation.

(*a*). 5 per cent. $MgSO_4$ extract. Vol. 100 cc. Precipitating salt was $MgSO_4$, added in substance.

Results:

Precipitate I. 5 grams = turbidity; 25 grams = flocculent precipitate.

Precipitate II. 35 grams = turbidity; 53 grams to saturation = flocculent precipitate.

Coagulations: Solution of precipitate I. 44°-47° C. (1).
Solution of precipitate II. 64° C. (3).

(*b*). Aqueous extract was treated with equal volume of a saturated solution of $(NH_4)_2SO_4$. The resultant precipitate was washed, dissolved in water, and the solution made faintly acid with acetic acid. On standing for some time a precipitate formed which corresponded to separation No. (1) of the coagulation series. This substance was filtered off, and the filtrate accurately neutralized. The neutral filtrate was used in (*c*) and (*d*) below.

(*c*). Neutral filtrate obtained in (*b*). Volume 100 cc. Precipitating salt $MgSO_4$.

Results:

Precipitate I. 20 grams = turbidity; 42 grams = flocculent precipitate.

Precipitate II. 43 grams = turbidity; 50 grams = flocculent precipitate.

Precipitate III. 56 grams = turbidity; 63 grams = flocculent precipitate.

Precipitate IV. 73 grams = turbidity; saturation + acid = final precipitate.

Coagulations: Solution of Precipitate I. 51°-58° C. (2); 65°-67° C. (3).
Solution of Precipitate II. 68°-69° C. (3).
Solution of Precipitate III. 66°-67° C. (3).
Solution of Precipitate IV. 54°-56° C. (2); 67°-70° C. (3).

(*d*). Neutral filtrate obtained in (*b*). Volume 100 cc. Precipitating salt was $(NH_4)_2SO_4$ added in saturated solution.*

* In this series, the method of performing the experiment was the same as in those just described, except that instead of weighed quantities of the salt, measured amounts of a saturated solution were added. The figures represent the volumes of this saturated $(NH_4)_2SO_4$ solution added to 100 cc. of the fluid under observation. A determination of the exact strength of the $(NH_4)_2SO_4$ solution showed it to contain 53.67 per cent. of that salt.

Results:

Precipitate I. 65 cc. = turbidity; on standing flocculent precipitate.

Precipitate II. 82 cc. = precipitate.

Precipitate III. 91 cc. = precipitate.

Precipitate IV. 100 cc. = precipitate.

At this point, according to differences in the precipitation reactions of albumins and globulins, all of the globulin-like substances should have been removed (the fluid now being half saturated with $(NH_4)_2SO_4$). Continued addition of saturated solution of $(NH_4)_2SO_4$ brought about further precipitation as follows:

Precipitate V. 125 cc. = precipitate.

Precipitate VI. 142 cc. = precipitate.

Precipitate VII. 150 cc. = precipitate.

No further precipitation was obtainable even on saturation with $(NH_4)_2SO_4$.

Coagulations:	Solution of Precipitate	I.	61°-63° C.	(3).
	Solution of Precipitate	II.	66°-67° C.	(3).
	Solution of Precipitate	III.	66°-67° C.	(3).
	Solution of Precipitate	IV.	56°-58° C.	(2).
	Solution of Precipitate	V.	53°-59° C.	(2).
	Solution of Precipitate	VI.	56°-57° C.	(2);
			64°-68° C.	(3).
	Solution of Precipitate	VII.	58°-60° C.	(2);
			67°-70° C.	(3).

A study of these results shows that among the proteid substances obtainable from the ligament by aqueous or saline extraction is one which may be separated by three different methods, *viz*: either by heating the extract to 40° C. after the addition of a trace of acetic acid, by the addition of $MgSO_4$ to the $MgSO_4$ extract in the proportion of 25 grams of salt to 100 cc. of extract or, finally, by faint acidification with acetic acid of the watery solution* of the precipitate obtained by half saturation of the aqueous extract with $(NH_4)_2SO_4$. Subsequent experiments (see page 13) showed that it could be separated from the aqueous extract by a slightly stronger percentage of acetic acid at room temperature.

A second substance, presumably a globulin, was precipitated

* Complete solution of this precipitate is brought about by the aid of the $(NH_4)_2SO_4$ mechanically adhering to it.

from the $MgSO_4$ extract by the addition of 53 grams of $MgSO_4$ to 100 cc. of extract. It coagulated at 65° C. This substance apparently also separates from the aqueous solution of the precipitate obtained by half saturation of the aqueous extract with $(NH_4)_2SO_4$ on the addition of $MgSO_4$ in quantities varying from 20 grams per 100 cc. up to the saturation point. It may also be obtained from such a solution by the addition of from 65 to 150 cc. of saturated solution of $(NH_4)_2SO_4$ to 100 cc. of fluid.

The third substance which we have identified coagulates at 56° C. and is precipitated by the addition of 42 grams of $MgSO_4$ to 100 cc. of its solution. A precipitate having the same coagulation temperature may be obtained by the addition of from 73 grams of $MgSO_4$ to 100 cc. of proteid solution up to the saturation equivalent. It is also precipitated by 100-150 cc. of saturated $(NH_4)_2SO_4$ solution added to 100 cc. of proteid solution.

Of these three substances, the one separating at the lowest temperature is not a coagulum. It is precipitated, not coagulated, by the action of the heat plus acid and consists of a nucleoproteid. (See page 13). The proteid which coagulates at 65° C. is also peculiar. Kauder[25] has shown that the globulins of the serum are completely precipitated by the addition of 92 cc. of saturated $(NH_4)_2SO_4$ solution to 100 cc. of proteid solution, while albumins do not begin to separate till 128 cc. have been added. If this substance consists of a single proteid it must, as far as its behavior toward $(NH_4)_2SO_4$ is concerned, partake of the nature of both an albumin and a globulin. Since, however, its precipitation is interrupted (see (*d*) above, Precipitates IV and V) it is more natural to suppose that we have an admixture of two proteids having approximately the same temperature of coagulation. The two proteids having the coagulation temperature of 64°-66° C. are fibrinoglobulin and serum albumin. It is probable that this substance is a mixture of two substances similar to these.

The proteid which coagulates at 56° C. is comparable as to its coagulation temperature to fibrinogen.

The two other proteids the presence of which is shown in A and B coagulate at about 75° C. and 82° C. respectively. They correspond very closely to the albumins ("serins") found in oxserum serum by Halliburton which coagulate at 77° C. and 84° C.

The quantity of these proteids appears to us to be too great to admit of the assumption that it is derived entirely from residues of serum left in the tissue. It is however, impossible we believe to remove every trace of serum from such a tissue without affecting its contents.

The method of fractional heat coagulation which we have used throughout these experiments has been the object of some criticism. Devised by Halliburton it was employed by him in a study of the proteids of blood serum.[17] It was afterwards used by Corin and Berard[10] in an investigation of the proteids of the egg-white. The results of these observers were doubted by Haycraft and Duggan[19] who criticized the method on the following grounds: first, that the coagulation temperature of a proteid is raised by dilution of its solution and that removal of a portion of the proteid in a coagulated form so lessens the amount of that remaining in solution as to effect a rise in its coagulation temperature; second, that the acidity of the solution is decreased by the separation of a coagulum, in this way also raising the temperature of coagulation; and finally, that the long heating necessary to remove all of the proteid from solution induced changes within the proteid itself which might alter its coagulation point. These conclusions have been combated and the method accepted by L. Fredericq,[15] Chittenden and Osborne[8] and others. Hewlett[21] however, under Halliburton's direction, has made a thorough study of the conditions affecting heat coagulation. He found that if the heating is conducted slowly, dilution does not affect the temperature of coagulation; also that prolonged heating does not bring about sufficient change in a proteid to influence its coagulation point. He therefore concludes—and we believe that we are justified in accepting his conclusion—that under standard conditions the coagulation temperature of any proteid is constant, and that with due regard for the maintenance of such conditions the identification of different proteids in a solution is possible by means of their fractional coagulation.

In all of our experiments on the proteids of this form of elastic tissue, every effort has been made to keep the conditions which are known to influence coagulation temperature, such as degree of acidity and salt content, constant. While we can not claim to

have made an unquestionable identification of all of the proteids in the ligamentum nuchae, we believe we have shown the presence of at least five of those substances in that tissue—the amount and character of which are such as to indicate that the metabolism in the tissue is of greater importance than has hitherto been supposed.

III. NUCLEOPROTEID.

We believe that the substance which we have separated from all extracts of the ligament by heating to 40° C. is a nucleoproteid. That it is not coagulated at this temperature is shown by the fact that it readily dissolves in dilute sodium carbonate. On slight acidification of its solution it is reprecipitated on standing at room temperature.

If 100 cc. of the aqueous extract of ligamentum nuchae be treated with 0.5 cc. of 36 per cent. acetic acid a flocculent precipitate gradually settles out. This precipitate is found to dissolve easily in dilute alkali. It does not coagulate when its acid or neutral solutions are heated. When fused with sodium hydroxide and potassium nitrate a distinct reaction for phosphorus may be obtained with molybdic solution. Further, after a very large amount of the aqueous extract of the tissue had been evaporated to small bulk on the water bath and the heavy precipitate of coagulated proteid filtered off, the addition of a few drops of 36 per cent. acetic acid to the filtrate gave a very heavy precipitate. This precipitate was readily soluble in 5 per cent. NaCl solution and was reprecipitated by saturation with the same salt. Its solutions would not coagulate in any medium. The substance contained phosphorus in organic combination.

Various forms of proteid matter are precipitable on acidification of their solutions. Of special interest to us are globulins, glucoproteids (mucoids) and nucleoproteids. Globulins, like serum globulin and fibrinogen, are precipitated by the minutest quantities of acid but are readily soluble in slight excess of such acids as acetic or hydrochloric. The connective tissue glucoproteids, *i. e.*, the mucoids, are precipitated by acid. They however are insoluble in a comparative excess of the acid used for precipitation. Further, the mucoids on decomposition by boiling with a mineral

acid yield a large proportion of a substance capable of reducing Fehling's solution. A careful series of tests on the substance which we have obtained on acidification of aqueous ligament extracts shows that it cannot belong to either of these two classes of proteids. That it can not be a globulin is shown by the fact that it is insoluble in slight excess of acetic acid and that it does not coagulate on heating its solutions. That it can not be mucoid is evidenced by the observation that, on decomposition with acid, it does not give more than a trace of a reduction of Fehling's solution. Furthermore, it shows a decided tendency to redissolve in a slight excess of hydrochloric acid. That the substance consists of a true nucleoproteid the following experiments will show.

A special preparation has been made as follows: The aqueous extract of 8 kilos of ligament was obtained as described on page 6, and to it was added 0.5 cc. of 36 per cent. acetic acid per 100 cc. of fluid. The flocculent precipitate which formed on standing was dissolved in 0.3 per cent. Na_2CO_3 and the solution filtered. The filtrate was water clear. This fluid was neutralized accurately with acetic acid, and then excess acetic acid added till precipitation occurred. From 1 to 1.3 cc. of 36 per cent. acetic acid per 100 cc. of the neutral fluid was required as a rule to bring about precipitation—a strength of acid in which globulins would readily dissolve. The precipitate was carefully washed, again dissolved and reprecipitated in a similar manner. It was finally washed free from acid and extracted in the usual way with alcohol and ether. The dry product from 8 kilos of ligament weighed 4.5 grams (0.056 per cent. of the fresh tissue).

Analysis of this product gave the following results for the percentage of phosphorus in the ash-free substance: (1) 0.54; (2) 0.50; average 0.52. The phosphorus in the ash in two duplicate determinations was found to amount to 0.05 per cent. of the dry proteid. Therefore even if we assume that all of the phosphorus of the ash arises from inorganic impurities, there is still an average amount of phosphorus in organic combination amounting to 0.47 per cent.

2 grams of the purified substance were decomposed by boiling with 50 cc. of 10 per cent. hydrochloric acid for twenty minutes. The fluid was neutralized with sodium hydroxide, acidified with

acetic acid and allowed to stand for twenty-four hours. No guanin separated. The fluid was then made alkaline with ammonia and treated with ammoniacal silver solution. A fairly heavy precipitate formed which was filtered off and washed. It was then dissolved in a little boiling nitric acid (sp. gr. 1.1) after the addition of a little urea and filtered while hot. The filtrate deposited no crystals on cooling, indicating the absence of hypoxanthin. On neutralization of the cold filtrate with ammonia, a precipitate was obtained apparently equal in bulk to the one first produced by addition of ammoniacal silver solution. That this precipitate consists of xanthin silver was proved by its method of preparation and by the fact that it gave a very beautiful reaction with Fischer's[14] modification of Weidel's test for xanthin

According to the generally accepted views,[41] all nucleoproteids contain within their molecules carbohydrate radicals. We have endeavored to detect the presence of such radicals in the following manner.

1 gram of the purified substance was decomposed by heating for three hours with 2 per cent. hydrochloric acid. The neutralized mixture was tested with Fehling's solution. On standing over night a very slight amount of cuprous oxide settled out. Furthermore, on boiling a small amount of the substance with hydrochloric acid in the presence of phloroglucin, no cherry-red color is apparent. Examination of the amyl alcohol extract of this decomposition mixture with the spectroscope, however, shows an absorption band between the D and E lines of the spectrum.

On the basis of these experiments we feel confident that in this substance we have a nucleoproteid containing within its molecule radicals which may be split off as proteid, carbohydrate, nuclein base and phosphoric acid.

Though the nucleoproteids as a class have come to be associated with the more purely cellular tissues, the fact remains that they are the essential constituents of every cell nucleus and must therefore exist in every living tissue. The nucleoproteid of the ligament appears to be comparable, as regards its phosphorus content, to that found by Pekelharing[48] in the muscle, which contains 0.7 per cent of phosphorus.

IV. MUCOID.

Since the discovery of mucoid among the proteids of tendon by Rollett,[45] only the most indefinite statements regarding the occurrence of this substance in elastic tissue are to be found. Kühne[30] some years later in his discussion of elastic tissues assumes its presence in this form of tissue. Neumeister,[42] much more recently, apparently regards its existence in all forms of connective tissue as certain, but refers to no experimental evidence as to its detection in the elastic tissues. Up till the time when this work was begun the only observation which bears on this question was that of Krakow[29] who by means of Schmiedeberg's method succeeded in obtaining chondroitin sulphuric acid from the ligamentum nuchae. The very recent work of Levene[31] has shown the possibility of obtaining chondroitin sulphuric acid as a cleavage product of tendon mucoid. Therefore Krakow's discovery may be regarded in the light of more recent facts as evidence of the presence of mucoid in the ligament.

In our preliminary experiments we found that the amount of mucoid which could be extracted from the ligamenta nuchae was very considerable. Vandegrift and Gies[50] have since found the average quantity to equal 0.525 per cent. of the fresh and 1.237 per cent. of the dry tissue—a lower percentage than exists in tendon.[4]

Our preparations were made by the method of Chittenden and Gies.[6] Quantities of the ligament varying from three to nine kilos were employed. The substance was purified by redissolving in 0.05 per cent. KOH or half-saturated lime water and reprecipitating by acid several times; also by thorough washing in alcohol and ether.

The products obtained in this manner have not been subjected to complete elementary analysis. The following facts however show its close relationship to the other connective tissue mucoids.[33]

In physical appearance the purified product is practically the same as tendomucoid or osseomucoid. It gives the color reactions of the proteids. On decomposition by boiling with 2 per cent. hydrochloric acid it yields a reducing substance and ethereal sulphuric acid. The reducing substance forms phenyldextrosazone-like crystals with phenylhydrazine, indicating the presence of

glucosamine among the hydration products. Among the other substances resulting from its hydrolytic decomposition are an anti-albumid-like body, acid albuminate, proteoses, and peptone. It is digestible in pepsin-hydrochloric acid and leaves a residue containing considerable reducing substance. Its sulphur may be obtained both as sulphate and sulphide. The pure substance does not contain phosphorus.

Ligament mucoid is soluble in 0.05 per cent. sodium carbonate solution, half-saturated lime-water and 5 per cent. sodium chloride solution. It is insoluble in 0.1 per cent. hydrochloric acid but is somewhat soluble in 0.2 per cent. solution of the same. It is acid to litmus, neutralizes dilute alkali, and has the same general precipitation reactions as the other connective tissue mucoids. No chlorine was detected in any of our preparations.

The percentage amounts of nitrogen, total sulphur, and sulphur in ethereal combination affords a favorable basis of comparison of this with the mucoids from other sources. The results on ligament mucoid are given in the table below. The usual amounts of substance, dried to constant weight at 100°-110° C. were taken for determination. The ash of our preparations varied between 1.04 and 1.90 per cent., most of which consisted of calcium and phosphoric acid. The phosphorus in the ash of preparation B amounted to 0.16 per cent. of the proteid, while the total percentage of phosphorus in the proteid was 0.18 per cent. The figures given are calculated on ash-free substance.

Preparations.	A	B	C	D	E	General averages.
Nitrogen	12.80	13.40	13.74	13.90	13.27	
"	13.01	13.64	13.66	13.82	13.22	
"	12.90	13.52	13.70	13.86	13.25	13.44
Total sulphur..	2.05	1.77	1.49	1.37	1.45	
" ..	2.09	1.68	...	1.27	1.40	
" ..	2.07	1.73	1.49	1.32	1.42	1.61
Sulphur as SO_3	1.32	1.02	0.90			
"	1.17	...	...			
"	1.25	1.02	0.90			1.06

Direct comparison is made in the following summary.

Substances.	Ligament mucoid.	Chondro-mucoid.[24]	Tendo-mucoid.[11]	Osseo-mucoid.[18]
Nitrogen	13.44	12.58	12.47	13.49
Total sulphur.....	1.61	2.42	2.20	1.85
Sulphur as SO_3.....	1.06	...	1.46	1.04

It will be noted that the nitrogen content of ligament mucoid appears to be higher than that of chondromucoid and of tendomucoid; its sulphur content at the same time is somewhat lower. In all the data obtained by us however it appears to be very comparable to osseomucoid. In making any such comparison however it must be remembered that the evidence which we have regarding these substances seems to show that more than one mucoid may be present in any one tissue. Variability in the composition of the substances as they are separated may therefore be due to variations in the proportion of the mixture of these mucoids. We have every reason to believe that the same deduction may be made with regard to the mucoid which we have prepared from ligament. At any rate we are justified in the conclusion that ligament mucoid is very similar to, if not identical with, the other connective tissue mucoids.

V. ELASTIN.

The discovery of these comparatively large amounts of coagulable proteids, nucleoproteid, and mucoid made desirable a study of the influence which the presence of these proteids might exert on the purity of elastin as it has hitherto been prepared and hence also on the results of its investigation.

Preparation. Historical.—The methods which have been used up to the present time for the preparation of elastin are as follows.

Tilanus,[49] who was the first to analyze elastic tissue, extracted the ligamentum nuchae of the cow with cold water, then with alcohol and ether. His product by this method was little better than prepared ligament. In a second preparation he added extraction in boiling dilute acetic acid. By this process he probably eliminated most of the coagulable proteid and collagen but undoubtedly left in the tissue a large proportion of the nucleoproteid and mucoid. The impure residue which he obtained he considered to be an individual substance—elastin—and as the result of his analyses he ascribed to it the formula $C_{52}H_{80}N_{14}O_{14}$. Unlike the product obtained by his first method, the latter substance contained no sulphur.

W. Müller[39] improved Tilanus' methods by adding treatment with boiling dilute alkali and cold dilute mineral acid to the process. He boiled the tissue alternately in dilute acetic acid and in dilute potassium hydroxide, and then extracted in cold dilute hydrochloric acid. This treatment if long enough continued would undoubtedly remove the other elements of the tissue but is also very favorable to decomposition of the elastin.

Horbaczewski[28] introduced into the method of Müller repeated extraction of the ligament with boiling water. This step, however, while facilitating removal of collagen rendered solution of coagulable proteid more difficult since it brought about their coagulation within the meshes of the tissue. It does not in any way decrease the chances of decomposing the elastin.

Chittenden and Hart[7] recognized the possibility of inducing alteration in the nature of the elastin by such means, and made a comparative study of the elastin prepared by Horbaczewski's method with that made according to a similar method which did not include boiling with alkali. Elastin prepared without the aid of alkali contained 0.3 per cent. of sulphur, while that which had undergone long extraction with 1 per cent. potassium hydroxide with final boiling in the same fluid was sulphur-free. These authors are inclined to believe that elastin contains sulphur which is split off by Horbaczewski's method of treatment. But although they exclude the decomposing action of potassium hydroxide by omitting treatment with it, they did not substitute any means which would exert a similar solvent effect on the nucleoproteid and mucoid.

Bergh[2] has recently obtained elastin from the cervical ligament by Horbaczewski's method, adding however digestion in pepsin-hydrochloric acid. Elastin however is readily soluble in gastric juice, so that this addition could hardly have done more than bring about increased change in the elastin itself. Aside from the determination of sulphur in his elastin and in Grübler's elastin (a commercial product made by the method of Horbaczewski) Bergh made no attempt to ascertain the elementary composition.

The ligament elastins investigated by other observers, whose analytic results are given below, were all from the same source, *viz*: the ligamentum nuchae of the ox. Morochowetz[37] made his

products by Müller's method; Stohmann and Langbein[48] by Horbaczewski's; Zoja,[52] Mann,[32] and Eustis[9] each used method of Chittenden and Hart.

A summary of the results on the average percentage composition of elastin prepared by the methods just outlined. The figures are calculated on ash-free products.

Method of preparation.	C	H	N	S	O
TILANUS:					
(*a*) Not extracted with acid	54.65	7.26	17.41	0.34	20.34
(*b*) Extracted with acids	55.65	7.41	17.74	...	19.20
MÜLLER:*					
Extracted with hot alkali	55.46	7.41	16.19	...	20.94
HORBACZEWSKI:					
Extracted with hot alkali	54.32	6.99	16.75	...	21.94
MOROCHOWETZ:†					
Extracted with hot alkali	?	?	?	0.63	?
CHITTENDEN AND HART:					
(*a*) Prepared by Horbaczewski's method	54.24	7.27	16.70	...	21.79
(*b*) Their own without extraction in alkali	54.08	7.20	16.85	0.30	21.57
STOHMANN AND LANGBEIN:					
Extracted with hot alkali	55.03	7.20	16.91	0.18	20.68
ZOJA:					
Not extracted with hot alkali	?	?	16.96	0.28	?
MANN:					
Not extracted with hot alkali	?	?	16.52	?	?
EUSTIS:‡					
Not extracted with hot alkali	54.70	7.40	16.65	0.14	21.11

It will be observed from this summary, that, in general, extraction with hot alkali resulted in the production of sulphur-free products. With few exceptions, methods which did not include such extraction gave elastins containing sulphur.

* Müller found 0.08 per cent. of sulphur in his elastin, which he assumed to be due to accidental impurity.

† It has been shown by Chittenden and Hart that in elastoses there is a diminution in the content of carbon and an increase in the content of oxygen. Morochowetz's analysis of his elastose gave the following results:

C	H	N	S	O
55.90	7.29	16.68	0.62	19.50

He did not completely analyze his original elastin, except for sulphur, with the result given above. We believe from these facts that his elastin was impure, and the figures for sulphur were inaccurate.

‡ Eustis made only a partial analysis. We wish here to thank him for a sample on which we obtained the remaining results. The individual ash-free determinations were as follows:

C	H	N	S
54.32	7.32	16.66	0.15
54.52	7.47	16.64	0.12

On testing the alkaline fluid obtained in their preparation of elastin by Horbaczewski's method, Chittenden and Hart found distinct evidence of hydrogen sulphide. Whether this sulphur originates in loosely combined sulphur within the elastin molecule, or in other proteids than elastin, the presence of which was not excluded by their method of preparation, these authors do not decide.

The solution of this problem we have sought by means of introducing into the method of Chittenden and Hart a solvent for all the sulphur-containing proteids which would not however have any decomposing action. The only constituents of elastic tissue so far as we know which require alkali for their solution are mucoid and nucleoproteid. These substances as they are situated within the tissue resist the solvent effect of boiling water or acid. Furthermore the preliminary boiling with water first used by Horbaczewski and afterwards by Chittenden and Hart must undoubtedly result in the coagulation of a large proportion of the simple proteids within the meshes of the tissue, thereby transforming them into a form of proteid which requires decomposition for solution.

Improved Method.—Our method for the preparation of ligament elastin includes extraction with cold half-saturated lime-water instead of destructive boiling with potassium hydroxide.

The cervical ligaments from freshly killed cattle were carefully freed from all adherent fat and connective tissue membranes and cut into longitudinal strips. These strips after a preliminary washing with cold water were put through a meat chopper* several times. The resulting hash was thoroughly extracted in distilled water many times renewed. The finely divided tissue was next extracted repeatedly with large volumes of cold half-saturated lime water. After four extractions, test portions of the fluid were found to give no turbidity on acidification. The alkali was completely washed out of the tissue by cold water, after which it was boiled in water till only traces of dissolved proteid (elastoses) could be detected in the washings. The tissue was then subjected to treatment with 10 per cent. acetic acid for several hours. After

[1] In none of the previous investigations of this albuminoid is any mention made of any special effort to obtain the tissue in as finely divided a condition as possible. We believe this to be a very important step in the process, as some of our results indicate. See pages 22 (note) and 27.

partially washing out the acetic acid with water it was put into 5 per cent. hydrochloric acid and allowed to stand at room temperature for several hours. The acid was then completely removed by washing in water. Finally the tissue was dehydrated by thorough extraction with boiling alcohol and ether, a process which removed all adherent fatty bodies.*

The elastin particles thus prepared were soft and porous and could easily be ground to a very light cream-colored powder. Under the microscope the particles were found to consist of typical elastic fibres in the meshes of which no extraneous matter could be detected.

In order to study the effect of the above modified method of preparation and to obtain further information concerning the composition of elastin we have made several samples of elastin both by the Chittenden and Hart method as well as by our own. These products have been subjected to careful comparative analysis by the methods recently described by Hawk and Gies.

Elementary Composition. Preparation No. 1.—Preparations 1-4 were made by the Chittenden and Hart process as follows: Finely minced tissue (100 grams) was boiled in water until practically nothing more dissolved.† This process required about ten changes of 1 litre of water and a total of seventy-five hours for completion. The substance was next warmed in 1 litre of 10 per cent. acetic acid for one and one-half hours on a water-bath. It was kept in the same fluid eighteen hours longer at room temperature and then boiled for four hours directly over a flame. The acid was then thoroughly washed out and the substance kept in five per cent. hydrochloric acid for eighteen hours at room temperature. After the mineral acid had been thoroughly removed the treatment in the acids, with appropriate washing, was repeated. Finally, dehydration and removal of fat and extractive matter were effected in boiling alcohol-ether in the usual manner. The analytic results follow:

* Further details will be found with the records of analysis. See pages 22 and 24.

† One variation from the Chittenden and Hart method is to be noted. We brought about a fineness of division of the tissue by means of a hashing machine, whereas they chopped the tissue with a knife. We believe our method led to a much more thorough extraction.

Carbon and Hydrogen. 0.2909 gram substance gave 0.5752 gram CO_2 and 0.1906 gram H_2O = 53.93 per cent. C and 7.28 per cent. H; 0.2538 gram substance gave 0.5078 gram CO_2 and 0.1659 gram H_2O = 54.56 (?) per cent. C and 7.26 per cent. H; 0.2603 gram substance gave 0.5159 gram CO_2 and 0.1703 gram H_2O = 54.05 per cent. C and 7.27 per cent. H; 0.2591 gram substance gave 0.5118 gram CO_2 and 0.1681 gram H_2O = 53.87 per cent. C and 7.21 per cent. H.

Nitrogen. 0.2909 gram substance gave 0.0469 gram N = 16.12 per cent. N; 0.3527 gram substance gave 0.0565 gram N = 16.01 per cent. N.

Sulphur. 1.2540 grams substance gave 0.0153 gram $BaSO_4$ = 0.17 per cent. S; 0.9790 gram substance gave 0.0141 gram $BaSO_4$ = 0.20 per cent. S; 0.6661 gram substance gave 0.0119 gram $BaSO_4$ = 0.25 per cent. S.

Ash. 0.4504 gram substance gave 0.0038 gram ash = 0.84 per cent. ash; 0.3424 gram substance gave 0.0025 gram ash = 0.73 per cent. ash.

PERCENTAGE COMPOSITION OF THE ASH-FREE SUBSTANCE.

										Average.
C	54.35	...	54.48	54.30	...	...	...	...	...	54.38
H	7.34	7.32	7.33	7.27	...	...	...	...	...	7.32
N	...	...	...	...	16.25	16.13	...	...	...	16.19
S	...	...	...	...	...	...	0.17	0.20	0.25	0.21
O	...	...	...	...	...	...	...	...	...	21.90

Preparation No. 2.

Carbon and Hydrogen. 0.2572 gram substance gave 0.5067 gram CO_2 and 0.1702 gram H_2O = 53.73 per cent. C and 7.35 per cent. H; 0.3730 gram substance gave 0.7383 gram CO_2 and 0.2408 gram H_2O = 53.98 per cent. C and 7.17 per cent. H; 0.4186 gram substance gave 0.2711 gram H_2O = 7.22 per cent. H; 0.4614 gram substance gave 0.9096 gram CO_2 and 0.2968 gram H_2O = 53.77 per cent. C and 7.15 per cent. H.

Nitrogen. 0.4863 gram substance gave 0.0811 gram N = 16.67 per cent. N; 0.2892 gram substance gave 0.0481 gram N = 16.62 per cent. N; 0.2521 gram substance gave 0.0426 gram N = 16.88 per cent. N.

Sulphur. 0.5535 gram substance gave 0.0089 gram $BaSO_4$ = 0.22 per cent. S; 0.7942 gram substance gave 0.0112 gram $BaSO_4$ = 0.19 per cent. S.

Ash. 0.5009 gram substance gave 0.0030 gram ash = 0.60 per cent. ash; 0.5364 gram substance gave 0.0031 gram ash = 0.58 per cent. ash.

PERCENTAGE COMPOSITION OF THE ASH-FREE SUBSTANCE.

										Average.
C	54.05	54.30	...	54.09	...	...	...	...	...	54.15
H	7.39	7.21	7.26	7.19	...	...	...	...	...	7.26
N	...	...	...	...	16.77	16.71	16.98	...	...	16.82
S	...	...	...	...	...	...	...	0.22	0.20	0.21
O	...	...	...	...	...	...	...	...	...	21.56

Preparation No. 3.

Carbon and Hydrogen. 0.2562 gram substance gave 0.5101 gram CO_2 and 0.1694 gram H_2O = 54.30 per cent. C and 7.35 per cent. H.

Nitrogen. 0.3305 gram substance gave 0.0550 gram N = 16.64 per cent. N; 0.3577 gram substance gave 0.0596 gram N = 16.67 per cent. N.

Sulphur. 1.1549 grams substance gave 0.0128 gram $BaSO_4$ = 0.15 per cent. S; 0.7953 gram substance gave 0.0100 gram $BaSO_4$ = 0.17 per cent. S.

Ash. 0.6690 gram substance gave 0.0045 gram ash = 0.67 per cent. ash; 0.5782 gram substance gave 0.0038 gram ash = 0.66 per cent. ash.

PERCENTAGE COMPOSITION OF THE ASH-FREE SUBSTANCE.

						Average.
C	54.67	...	...	...	...	54.67
H	7.40	...	...	...	...	7.40
N	...	16.75	16.78	...	...	16.76
S	...	...	...	0.15	0.17	0.16
O	...	...	...	...	...	21.01

Preparation No. 4.

Carbon and Hydrogen. 0.2571 gram substance gave 0.5084 gram CO_2 and 0.1671 gram H_2O = 53.93 per cent. C. and 7.22 per cent H.

Nitrogen. 0.3386 gram substance gave 0.0562 gram N = 16.59 per cent. N; 0.2545 gram substance gave 0.0426 gram N = 16.72 per cent. N.

Sulphur. 0.9068 gram substance gave 0.0163 gram $BaSO_4$ = 0.25 per cent. S; 1.0077 grams substance gave 0.0163 gram $BaSO_4$ = 0.22 per cent. S.

Ash. 0.4931 gram substance gave 0.0052 gram ash = 1.05 per cent. ash; 0.4412 gram substance gave 0.0050 gram ash = 1.13 per cent. ash.

PERCENTAGE COMPOSITION OF THE ASH-FREE SUBSTANCE.

						Average.
C	54.52	...	...	...	...	54.52
H	7.30	...	...	...	...	7.30
N	...	16.77	16.90	...	...	16.83
S	...	...	...	0.25	0.22	0.24
O	...	...	...	...	...	21.11

Preparation No. 5.—Preparations 5–8 were made by our own method. 100 grams of ligament strips were washed in cold running water 24-48 hours. The strips were next run through a hashing machine and the hash thoroughly extracted several times (for 3 days) in half-saturated lime-water. The last extract did not become turbid on acidification. The alkali was completely washed out of the hash with water. The rest of the process—boiling in water, etc.—was the same in time, order, and character that for preparations 1-4.

Carbon and Hydrogen. 0.2448 gram substance gave 0.4819 gram CO_2 and 0.1648 gram H_2O = 53.69 per cent. C and 7.48 per cent. H; 0.2627 gram substance gave 0.5142 gram CO_2 and 0.1776 gram H_2O = 53.38 per cent. C and 7.51 per cent. H; 0.4568 gram substance gave 0.8922 gram CO_2 and 0.2916 gram H_2O = 53.27 per cent. C and 7.09 per cent. H.

Nitrogen. 0.3735 gram substance gave 0.0620 gram N = 16.59 per cent. N; 0.2420 gram substance gave 0.0400 gram N = 16.51 per cent. N; 0.2498 gram substance gave 0.0417 gram N = 16.69 per cent. N.

Sulphur. 1.0358 gram substance gave 0.0119 gram BaSO = 0.16 per cent. S; 0.5907 gram substance gave 0.0075 gram $BaSO_4$ = 0.17 per cent. S.

Ash. 0.3943 gram substance gave 0.0029 gram ash = 0.74 per cent. ash; 0.3907 gram substance gave 0.0036 gram ash = 0.92 per cent. ash.

PERCENTAGE COMPOSITION OF THE ASH-FREE SUBSTANCE.

									Average.
C	54.14	53.83	53.72	...	...	...	...	...	53.90
H	7.54	7.57	7.15	...	...	...	...	...	7.42
N	...	...	...	16.73	16.65	16.83	...	...	16.74
S	...	...	...	...	...	...	0.16	0.17	0.16
O	...	...	...	...	...	...	...	...	21.78

Preparation No. 6.

Carbon and Hydrogen. 0.3285 gram substance gave 0.6550 gram CO_2 and 0.2161 gram H_2O = 54.38 per cent. C and 7.31 per cent. H; 0.2539 gram substance gave 0.5036 gram CO_2 and 0.1654 gram H_2O = 54.09 per cent. C and 7.24 per cent. H; 0.3343 gram substance gave 0.6662 gram CO_2 and 0.2202 H_2O = 54.35 per cent. C and 7.32 per cent. H.

Nitrogen. 0.4117 gram substance gave 0.0701 gram N = 17.02 per cent. N; 0.2965 gram substance gave 0.0510 gram N = 17.18 per cent. N; 0.2797 gram substance gave 0.0478 gram N = 17.08 per cent. N.

Sulphur. 1.3763 grams substance gave 0.0128 gram $BaSO_4$ = 0.13 per cent. S; 1.1255 grams substance gave 0.0121 gram $BaSO_4$ = 0.15 per cent. S.

Ash. 0.9620 gram substance gave 0.0008 gram ash = 0.08 per cent. ash; 1.0230 grams substance gave 0.0009 gram ash = 0.09 per cent. ash.

PERCENTAGE COMPOSITION OF THE ASH-FREE SUBSTANCE.

									Average.
C	54.43	54.14	54.40	...	...	...	...	...	54.32
H	7.32	7.25	7.33	...	...	...	...	...	7.30
N	...	...	...	17.03	17.20	17.09	...	...	17.11
S	...	...	...	...	...	...	0.13	0.15	0.14
O	...	...	...	...	...	...	...	...	21.13

Preparation No. 7.

Carbon and Hydrogen. 0.2584 gram substance gave 0.5120 gram CO_2 and 0.1685 gram H_2O = 54.04 per cent. C and 7.25 per cent. H.

Nitrogen. 0.4656 gram substance gave 0.0764 gram N = 16.42 per cent. N; 0.4482 gram substance gave 0.0744 gram N = 16.60 per cent. N.

Sulphur. 0.8678 gram substance gave 0.0096 gram $BaSO_4$ = 0.15 per cent. S; 0.8896 gram substance gave 0.0080 gram $BaSO_4$ = 0.12 per cent. S.

Ash. 0.5082 gram substance gave 0.0038 gram ash = 0.75 per cent. ash; 0.3540 gram substance gave 0.0030 gram ash = 0.85 per cent. ash.

PERCENTAGE COMPOSITION OF THE ASH-FREE SUBSTANCE.

						Average.
C	54.47	...	...	...	...	54.47
H	7.30	...	...	...	...	7.30
N	...	16.55	16.73	...	...	16.64
S	...	...	...	0.15	0.12	0.14
O	...	...	...	...	...	21.45

Preparation No. 8.

Carbon and Hydrogen. 0.2552 gram substance gave 0.5000 gram CO_2 and 0.1666 gram H_2O = 53.43 per cent. C and 7.25 per cent. H.

Nitrogen. 0.3169 gram substance gave 0.0536 gram N = 16.90 per cent. N; 0.4482 gram substance gave 0.0431 gram N = 16.84 per cent. N.

Sulphur. 0.8235 gram substance gave 0.0087 gram $BaSO_4$ = 0.15 per cent. S; 0.5679 gram substance gave 0.0059 gram $BaSO_4$ = 0.14 per cent. S.

Ash. 0.4533 gram substance gave 0.0032 gram ash = 0.71 per cent. ash; 0.3851 gram substance gave 0.0031 gram ash = 0.81 per cent. ash.

PERCENTAGE COMPOSITION OF THE ASH-FREE SUBSTANCE.

						Average.
C	53.84	...	...	...	...	53.84
H	7.31	...	...	...	...	7.31
N	...	17.03	16.96	...	...	17.00
S	...	...	...	0.15	0.14	0.14
O	...	...	...	...	...	21.71

The results for elementary composition of our eight preparations are brought together in the appended general summary. No great differences in the average composition of the preparations of each group are to be found. In fact the general analytic harmony is very striking and rather unexpected. The significant feature is to be seen in the figures for sulphur. The quantity is slight throughout, with the content of sulphur in preparations 5-8 regularly lower than that of preparations 1-4.

GENERAL SUMMARY OF ELEMENTARY COMPOSITION.

Elements.	Preparations 1–4 Made by the method of Chittenden and Hart. 1	2	3	4	Av.	Preparations 5–8 Made by the method of Richards and Gies. 5	6	7	8	Av.	Gen'l av.
C	54.38	54.15	54.67	54.52	54.43	53.90	54.32	54.47	53.84	54.14	54.29
H	7.32	7.26	7.40	7.30	7.32	7.42	7.30	7.30	7.31	7.33	7.33
N	16.19	16.82	16.76	16.83	16.65	16.74	17.11	16.64	17.00	16.87	16.76
S	0.21	0.21	0.16	0.24	0.21	0.16	0.14	0.14	0.14	0.14	0.18
O	21.90	21.56	21.01	21.11	21.39	21.78	21.13	21.45	21.71	21.52	21.44

The following summary affords ready comparison in this connection with related results for average elementary composition:

	C	H	N	S	O
Ligament elastin:					
Horbaczewski	54.32	6.99	16.75	....	21.94
Chittenden and Hart	54.08	7.20	16.85	0.30	21.57
Richards and Gies	54.14	7.33	16.87	0.14	21.52
Aorta elastin:					
Schwarz[47]	54.34	7.08	16.79	0.38	21.41
Bergh	53.99	7.54	15.20	0.60	22.67

Reactions.—We have little to add to what has already been noted in this connection. We have found that elastin is not as resistant to the action of acids and alkalies as has been supposed. The purified powdered substance is somewhat soluble in cold 0.2 per cent. hydrochloric acid on standing and is very readily soluble in 1 per cent. potassium hydroxide on warming. These facts suggest that the state of division of the tissue is a very important factor in the purification of the substance. We believe that the close agreement in composition of the two groups of our products was mainly due to the particularly fine division of the tissue employed. The agents used in the purification process were thereby given ready access to the interfibrillar material.

Sulphur Content.—As has been already pointed out, the elastins prepared by the older methods which included extraction with hot alkali were in most cases free from sulphur. Chittenden and Hart were the first to point out the danger in the use of this means, and found that elastin prepared without its use contained 0.3 per cent. of sulphur. This sulphur they believed existed in the elastin molecule. Their work has been confirmed by Zoja and Eustis.

Our results in this connection may be tabulated as follows:

Elastin made by the Chittenden and Hart method. Percentage of sulphur.*			Elastin made by the Richards and Giles method. Percentage of sulphur.*		
Number of preparation.	Direct determinations.	Average.	Number of preparation.	Direct determinations.	Average.
1	0.17 0.25 0.20	0.21	5	0.16 0.17	0.16
2	0.22 0.20	0.21	6	0.13 0.15	0.14
3	0.15 0.17	0.16	7	0.15 0.12	0.14
4	0.25 0.22	0.24	8	0.15 0.14	0.15
9†	0.16 0.18	0.17			
General average.....		0.20	General average.....		0.15

It will be seen that the average sulphur content of elastin made according to the older method was 0.20 per cent., whereas the elastins made by our own process by which the presence of mucoid, coagulable proteid, and nucleoproteid is as far as possible excluded, contain 0.15 per cent. of sulphur. The difference therefore in favor of our improved method amounts to 0.05 per cent. This difference though slight is constant. The greatest care was taken in making our preparations, the conditions to which both groups were subjected being as far as possible identical. Our results seem to indicate that sulphur, in minute quantity at least, is an integral part of pure ligament elastin.

Schwarz[47] has found that on boiling aorta elastin (prepared without the use of alkali) with 1 per cent. potassium hydroxide for four hours, all of the sulphur could be split off leaving an insoluble residue, free from sulphur, but *identical* in all its properties with the original elastin. Ligament elastin seems to be a different substance. On boiling samples of our own products with 1 per cent. potassium hydroxide no sulphide could be detected in the fluid by the addition of lead acetate. Furthermore our elastin was

* The ash of each preparation was slight in amount. The ash contained an appreciable proportion of sulphur—an average of 0.11 per cent. of the proteid of each group of preparations. This was doubtless derived in great part from the organic sulphur during incineration.

† This preparation was not completely analyzed, and therefore was not included in the series under elementary composition, page 104. It contained only 0.54 per cent. ash.

practically entirely dissolved within the first half hour of boiling. A sample of elastin prepared by Eustis according to the method of Chittenden and Hart gave a decided sulphur reaction when tested in a similar way.

These facts indicate that the small amount of sulphur contained in pure elastin is held in a form of combination which is not converted into sulphide by boiling with alkali.

Distribution of Nitrogen.—The discovery of the basic substances, arginin, histidin, and lysin among the cleavage products of proteid has thrown much new light on the inner make-up of proteid matter. The detection of these substances has been undertaken by Kossel and his co-workers among the decomposition products of a large number of proteids, and on the basis of the results Kossel[26] has stated his belief that on appropriate treatment all proteids would yield hexone bases. He has further expressed the opinion that all proteids may be looked upon as being built up about a basic (protamine) nucleus.

Until very recently elastin has not been studied in this regard. Bergh,[2] soon after Kossel had made the statements given above, attempted to isolate lysin and arginin from among the cleavage products of elastins prepared from the cervical ligament and from the aorta. His attempts failed. Hedin[20] by similar methods was unable to identify histidin. Their results would therefore indicate the absence of this group in the elastin molecule. Kossel and Kutscher[27,28] however by an improved method subsequently separated arginin and lysin in very small amounts from the decomposition products of ligament elastin, thus directly contradicting the conclusions of Bergh and Hedin. Schwarz had previously claimed to have obtained lysatinin (*i. e.*, arginin and lysin) from aorta elastin. The quantities found were very minute.

This lack of agreement between Bergh and Hedin on the one hand and Kossel and Kutscher and Schwarz on the other, led to the study by Eustis[9] under Chittenden's direction of the proportion of basic nitrogen which could be split off from elastin in the form of organic bases by boiling with hydrochloric acid in the presence of stannous chloride. Following the procedure adopted by Schultze,[46] Eustis made five experiments on the same preparation of elastin with the following divergent results:

0.86	17.69	15.57	6.50	15.14

These discordant data indicate that the hexone bases are obtainable from elastin but led to the conclusion that the method used for their estimation was unreliable for quantitative purposes.

The divergence of these results made it seem desirable for us to repeat his experiments on elastin prepared by our own method as well as that which he employed. The preparations which we have employed were Nos. 1, 3, and 7. The method of cleavage was the same as that used by Eustis. Decomposition took place in 20 per cent. hydrochloric acid in the proportion of 4 cc. of acid to 1 gram of elastin. 1 gram of stannous chloride was added to the mixture which was boiled in a reflux condenser for 96 hours. After separation of the tin, the determination of nitrogen as ammonia, etc., was carried out according to the method of Schultze.

Besides the estimation of these bodies in this indirect way we have made a decomposition of a large amount of elastin by the more recent method of Kossel and Kutscher[28] with a view to obtaining the hexone bases if possible and determining their percentage by weight. 100 grams of the pure elastin (Preparation No. 6) were boiled for 14 hours with 300 grams of sulphuric acid and 600 grams of water. A small portion of the decomposition mixture was subjected to indirect determination according to Schultze's method; the great bulk of it, however, was treated by the steps devised by Kossel and Kutscher for the isolation and determination of arginin, lysin, and histidin. The results of our indirect determinations are given in the following table:

Number of preparation.	Weight of ash-free elastin used.	Nitrogen.						
		Grams.				Percentage of total.		
	Grams.	Total.	Ammonia.	Non-amido acids.	Bases.	Ammonia.	Non-amido acids.	Bases.
3	13.4361	2.2371	0.0507	2.1351	0.0666	2.26	95.44	2.98
1	11.4472	1.8533	0.0434	1.8238	0.0420	2.34	98.42	2.26
7	11.5549	1.9227	0.0333	1.8311	0.0593	1.73	95.23	3.08
6	105.2000	18.1012	0.2572	17.0081	0.9153	1.42	93.96	5.06
		Average.....				1.94	95.76	3.34

It will be seen at once that though there are noticeable variations in our results, there is yet a striking agreement among them, even though two methods of decomposition and widely different quantities of elastin were used. This uniformity is in striking contrast to the disagreement of the results obtained by Chittenden and

Eustis. It is also to be noticed that the percentage of basic nitrogen is greatest in the decomposition which was made according to Kossel and Kutscher's method—a result in harmony with their observation that cleavage with sulphuric acid brings about a larger yield of hexone bases than with any other acid.

While the strictest accuracy can not be claimed for these results, we feel justified in concluding that a portion of the nitrogen of elastin may be split off in the form of hexone bases.

Further and more direct evidence was obtained by the separation of arginin and histidin from the decomposition mixture of elastin No. 6. In continuation of Kossel and Kutscher's method, the amount of each base was determined by estimation of the nitrogen in the solution which contained it, as well as by the weight of a characteristic salt after its separation from that solution.

Arginin was weighed as the neutral nitrate and histidin as the dichloride. The microscopic appearance of both was typical. Unusual difficulty was encountered in separating lysin in the form of the picrate. While the solution, which, according to the theory of the method, should have contained nothing but lysin gave nitrogen values corresponding to 6.65 per cent. of lysin we were unable to obtain the lysin picrate from the solution.

The results have been included for the sake of comparison in a table of similar results obtained by Kossel and Kutscher on a number of other proteids.

	Percentage of total nitrogen.				Percentage of weight of original substance.			
Substance.	Histidin.	Arginin.	Lysin.	Ammonia.	Histidin.	Arginin.	Lysin.	Ammonia
Ligament elastin	0.170	1.380	6.65(?)	1.375	0.027	0.197	....	0.287
Zein	1.410	3.760	0	13.530	0.810	1.820	0	2.560
Commercial gelatin	?	16.600	?	1.400	?	9.3	5-6*	0.300
Thymus histon..	1.790	25.170	8.040	7.460	1.210	14.360	7.700	1.660
Salmin	0	87.800	0	0	0	84.300	0	0

Our results on the ligament elastin by the two methods do not agree very closely. This fact may find its explanation in several circumstances. In the purification of the bases it is necessary to make repeated precipitations of barium sulphate, barium phosphotungstate, silver sulphide, etc. At almost every step a heavy pre-

* Approximate values.

cipitate requires quantitative washing. The possibility of error is very great. Furthermore our results show that in elastin the percentage of bases is very small. The percentage of other decomposition products is therefore correspondingly large—a condition which multiplies the sources of error in the determination of the bases.

In view of these results, however, we feel confident in the belief that a small amount of nitrogen may be split off from pure elastin in the form of hexone bases, and that histidin as well as arginin and lysin may be obtained. The amount of these bodies is less than that obtained from any of the proteids hitherto examined in this respect.

Is Elastin a "Fat-Proteid Compound"?—Nerking[40,44] has recently found that a number of proteids, among them paraglobulin, submaxillary mucin, etc., contain fat or fatty acid radicals which can not be obtained by extraction with ether until the proteid has been digested by pepsin hydrochloric acid. He did not examine elastin in this connection. We have tested some of our preparations with entirely negative results.

Samples of preparations Nos. 5 and 6 were used. Amounts of substance taken were 5.6747 grams of No. 5 and 8.7429 grams of No. 6. After two weeks' continuous extraction with anhydrous ether in a Soxhlet apparatus only 0.0015 gram of extractive material could be obtained from No. 5, and 0.0013 gram from No. 6. After digesting each with pepsin hydrochloric acid as Nerking did in carrying out Dormeyer's method and thoroughly extracting the digestive mixture with ether only 0.0017 gram of ether-soluble matter was obtained from No. 5 and 0.0013 gram from No. 6. Therefore in this double extraction process only 0.0032 gram (0.056 per cent.) of ether extract was obtained from No. 5 and only 0.0026 gram (0.03 per cent.) from No. 6. Since the pepsin used contained from 0.5 to 1.0 milligram of ether-soluble material, only the merest trace at most of a fat-like substance could have existed in either of these preparations. It is certain that the elastin prepared by the method we employed is neither a compound nor an admixture of proteid with fat or fatty acid.

Digestibility.—The products of the pepsin digestion of elastin

have been studied by Horbaczewski[28] and by Chittenden and Hart.[7]

Horbaczewski digested elastin for several days with pepsin-hydrochloric acid; after removing most of the acid by dialysis he evaporated the digestive fluid to dryness. From this mixture he separated two substances: The first, called by him "hemielastin", was separated by strong acidification with acetic acid in the presence of sodium chloride in saturation; the second was prepared by drying the solution of digestion-products, after removal of the hemielastin, by means of lead carbonate. This substance he called elastinpeptone.

Chittenden and Hart in a more elaborate study of the digestive products of this albuminoid found that Horbaczewski's "hemielastin" was in reality protoelastose and his elastin peptone was deuteroelastose. They were unable to detect any true peptone even when digestion had gone on under favorable conditions. Hydration with dilute acid also failed to form any peptone. Notwithstanding these facts, they seem to have assumed that it could be produced from elastin for they make this statement: "We propose, later, to attempt a study of elastinpeptone, using for this purpose the elastose just described, as well as elastin itself and more vigorous digestive fluids, both peptic and tryptic."

In the preceding experiments as to possible fat admixture it was necessary to digest our preparations of elastin with pepsin hydrochloric acid. They were found to digest readily, passing into solution when from 5 to 8 grams were mixed with 300 cc. of 0.2 per cent. hydrochloric acid plus 0.2 gram of pepsin—a result in harmony with the earliest observations of Etzinger.[12] At the end of twenty-four hours only a trace of turbidity remained in the fluid, showing the absence of antialbumid-like body. Cumulative power of combining with acid was noted as digestion went on as is the case with the proteoses and peptones in general.[5]

After the mixtures had been extracted with ether for the purpose described, digestion was allowed to go on for six weeks. At the end of that period the elastose precipitate obtained on saturating the neutralized fluid with ammonium sulphate was surprisingly large. Only a slight additional amount could be obtained by boiling while saturated in acid and alkaline reactions. After

filtering and removal of ammonium sulphate from the filtrate by alcohol and barium carbonate, a solution was obtained which gave a strong biuret reaction. The amount of peptone precipitable from this solution, though slight, was more than could have arisen directly or by autodigestion of the pepsin preparation used.

More detailed information is afforded by a special experiment in this connection. 8.15 grams of elastin (preparation No. 6) were digested in a solution of 900 cc. of 0.4 per cent. hydrochloric acid and 2 grams of very active pepsin. Bacterial action was prevented by the action of the acid and by the addition of toluol. Complete solution of the elastin took place in twenty-four hours. At the end of seventeen days the elastose was separated quantitatively. Ash-free, it weighed 7.43 grams, showing that at least 0.7 gram of the original elastin had been transformed into peptone.

In two similar experiments 8 grams of elastin at the end of forty-six days' digestion yielded only 1.38 grams of elastose and from 10 grams of elastin after digesting for seventy days less than 1 gram could be precipitated by ammonium sulphate.

These experiments show that while elastoses are especially resistant to further pepsin digestion, under most favorable conditions large amounts of true peptone may be formed. The precipitate obtained on saturation of the digestive mixture with ammonium sulphate was found to consist of primary and secondary proteoses. Judging from the reactions of solutions of the mixed proteoses the amount of protoelastose was relatively large. Horbaczewski and Chittenden and Hart observed that solutions of protoelastose became turbid on warming. Our solutions became turbid on heating even to 40° C. The clear concentrated solutions of the elastoses gave heavy precipitates with concentrated nitric acid, picric acid, potassio-mercuric iodide and other proteid precipitants. These precipitates were only partially soluble on heating. On adding the same reagents to dilute elastose solution the precipitates which formed at once dissolved on warming and reappeared on cooling, as is the case with the other proteoses. Excess of sodium hydroxide produced a precipitation of some of the elastose, which precipitate persists even after boiling the mixture.

These reactions appear to be due to protoelastose, a peculiar member of the proteose series.

Heat of Combustion.—The potential energy of the proteids expressed in calories varies from 5000 to 6000 small calories per gram of substance. Proteids like peptone and the mucoids which contain comparatively small amounts of carbon have the lowest combustion equivalents while proteids which have high carbon content have the highest combustion equivalents. The heat of combustion of any albuminous substance depends on amounts and combinations of the carbon and oxygen contained in it. The figures for the composition of elastin would suggest that its heat of combustion is relatively great.

Stohmann and Langbein[48] have made some determinations on the heat of combustion of elastin made by Horbaczewski's method. The combustion equivalent was found to average 5961.3 calories per gram of ash-free substance, the highest equivalent for any animal proteid.

We have made a few determinations of the heat of combustion of some of our products. The results given in the following table were obtained on a preparation made by Eustis according to Chittenden's method, on one made by us by the same method (No. 2) and on two made by us by our own method (Nos. 5 and 6). The table also includes Stohmann and Langbein's figures.

In giving these results, we wish to express our thanks to Prof. W. O. Atwater, in whose laboratory the determinations were made and to his assistants, Messrs. Swett and Osterberg, for experimental aid.

	Direct determinations. Heat of combustion. Small calories per gram.			Averages: calculated for ash-free substance. Percentage composition.					Heat of combustion. Small calories.	
Preparation.	I	II	Aver.	C	H	N	S	O	Per gram.	For substance containing 1 gm. carbon.
Eustis	5933	5947	5940	54.42	7.40	16.65	0.14	21.39	5960	10952
Prep. No. 2	5849	5821	5835	54.15	7.26	16.82	0.21	21.56	5870	10840
Prep. No. 5	5840	5871	5855	53.90	7.42	16.74	0.16	21.78	5904	10954
Prep. No. 6	5923	5909	5916	54.32	7.30	17.11	0.14	21.13	5967	10985
Average.....			5886	54.20	7.34	16.83	0.16	21.47	5925	10933
Stohmann and Langbein.....				55.03	7.20	16.91	0.18	20.68	5961	10832

The general relation of our results to those for other proteids is seen in the following summary:

Substance.	Average percentage composition. C	H	N	S	O	Heat of combustion. Small calories. Per gram.	For substance containing 1 gm. carbon.
Ligament elastin*.....	54.36	7.32	16.85	0.17	21.31	5932	10912
Various animal and vegetable proteids, not including gluco-proteids†	52.64	7.08	16.00	1.03	23.20	5711	10849
Connective tissue mucoids‡..... ...	47.43	6.63	12.22	2.32	31.40	4981	10505

VI. COLLAGEN (GELATIN).

All forms of connective tissue contain collagen. Eulenberg was the first to demonstrate the presence of collagenous fibres in the ligamentum nuchae by obtaining its hydration product, gelatin. Recently accurate determinations of its amount have been made with the discovery that 7.23 per cent. of the fresh tissue or 17.04 of the dry is made up of this substance. In spite of its presence in this tissue in such large amounts it has never so far as we can learn been subjected to analysis. Its comparison to the collagen from other sources is therefore based on superficial grounds.

The presence of so much elastin in the ligament makes the separation of collagen as such by Ewald and Kühne's[18] method of digestion with trypsin impracticable. In order to obtain some idea of its character however, we have transformed it into gelatin and separated and studied this.

Preparation of Ligament Gelatin.—After the cleaned ligament had been hashed with a meat chopper, washed with water and extracted with half-saturated lime-water, and the alkali thoroughly removed with water, the residual tissue was boiled for some time in distilled water. The filtrate was concentrated somewhat on a water-bath, and the gelatin precipitated from it by pouring it into a large excess of alcohol. The typical fibrous precipitate of gelatin was obtained in this way. This was redissolved in water and reprecipitated by alcohol several times, finally purified and dehydrated by treatment with alcohol and ether.

Elementary Composition.—The elementary analysis of one preparation gave the following results:

* The figures for ligament elastin are the averages of the results obtained by Stohmann and Langbein and in our own experiments.

† Averages obtained by Stohmann and Langbein.

‡ Averages obtained by Hawk and Gies.

Carbon and Hydrogen. 0.2324 gram substance gave 0.1372 gram H_2O = 6.56 per cent. H; 0.3773 gram substance gave 0.6860 gram CO_2 and 0.2250 gram H_2O = 49.59 per cent. C and 6.63 per cent. H; 0.3681 gram substance gave 0.6705 gram CO_2 and 0.2194 gram H_2O = 49.68 per cent. C and 6.62 per cent. H.

Nitrogen. 0.2867 gram substance gave 0.0501 gram N = 17.47 per cent. N; 0.3578 gram substance gave 0.0634 gram N = 17.72 per cent. N.

Sulphur. 0.7370 gram substance gave 0.03050 gram $BaSO_4$ = 0.568 per cent. S; 0.9417 gram substance gave 0.03734 gram $BaSO_4$ = 0.544 per cent. S.

Ash. 0.3503 gram substance gave 0.0058 gram ash = 1.65 per cent. ash; 0.2746 gram substance gave 0.0047 gram ash = 1.71 per cent. ash.

PERCENTAGE COMPOSITION OF THE ASH-FREE SUBSTANCE.

								Average.
C	...	50.44	50.53	...	...	...	...	50.49
H	6.67	6.74	6.73	...	...	...	...	6.71
N	...	...	...	17.77	18.02	...	...	17.90
S	...	...	...	...	...	0.58	0.56	0.57
O	...	...	...	...	...	...	...	24.33

The following summary of percentage elementary composition shows the relation of ligament gelatin to bone and tendon gelatin, also to gelatin from corneal collagen and cartilage collagen:

	C	H	N	S	O
Ligament gelatin.....	50.49	6.71	17.90	0.57	24.33
Tendon gelatin[51].....	50.11	6.56	17.81	0.26	25.24
Bone gelatin[28].....	50.40	6.64	18.34	...	24.64
Corneal gelatin[25].....	...	...	16.95	0.30	...
Cartilage gelatin[26].....	...	...	16.14	...	...

A comparison of these figures indicates that there may be three groups of collagens, distinguished by difference in elementary composition. The group of which cartilage collagen is a type yields a gelatin containing 16 per cent. of nitrogen. Corneal gelatin contains about 17 per cent., while the gelatins obtained from tendon, bone, and ligament yields approximately 18 per cent. of nitrogen. Our results indicate that the collagen from the ligamentum nuchae of the ox is essentially the same as that in tendon and bone.

Heat of Combustion.—In two determinations of the heat of combustion of ligament gelatin we obtained an average of 5276 small calories (5261, 5291) as the combustion equivalent. These

figures accord very well with those previously obtained by other observers for different gelatins, as will be seen from the following summary, which gives also the combustion equivalents of two proteids having equivalents among the very lowest for albuminous substances:

Substance. Dried at 100°–110° C.	Heat of combustion. Small calories. Per gram.	Heat of combustion. Small calories. For substance containing 1 gm. of carbon.	Percentage composition. Carbon.	Percentage composition. Oxygen.
Ligament gelatin.....	5276	10450	50.49	24.33
Fish gelatin[3]..... ...	5242	10800	48.53	25.54
Commercial gelatin[1].....	5270	.....	....	....
Fibrin peptone[46].....	5299	10577	50.10	25.79
Tendomucoid[11].....	5003	10415	48.04	30.62

VII. CRYSTALLINE EXTRACTIVES.

In our first report of this work attention was called to the fact that ox ligament contains an appreciable amount of crystalline extractives. The only one the identity of which had been determined at that time was creatin, although we had obtained evidence of the presence of one or more of the nuclein bases. More definite results have since been acquired in a continuation of this work.

Preparation of Extract.—15 to 20 pounds of ligamenta nuchae which were perfectly fresh and contained only slightest traces of blood were finely minced in a meat chopper. The hash was extracted in a moderate amount of water for twelve to twenty-four hours, putrefaction being prevented by use of powdered thymol. This extract was strained through cloth, heated to boiling, and sufficient acid added to coagulate the proteids and precipitate mucoid or nucleoproteid. The precipitate so obtained was perfectly colorless, indicating the absence of haemoglobin from the extract.

The slightly acid filtrate was then neutralized and evaporated on the water-bath to a thin syrup. This concentrated extract presented all the physical qualities of ordinary "beef extract." It contained a trace of proteid matter (probably derived gelatin or albuminate) but no reducing substance could be detected in it. In one sample leucin and tyrosin were detected, originating probably in the hydration of the small amount of dissolved proteid taking place in the process of boiling and evaporation. Creatinin was

also detected, arising undoubtedly from the hydration of creatin. Chloride and phosphate of sodium and calcium were present in comparative abundance. Sulphate was also present.

Creatin.—The concentrated extract was diluted with several volumes of water and treated with lead acetate for the removal of inorganic radicals. The excess of lead was precipitated by hydrogen sulphide and the filtrate evaporated to a thin syrup on the water-bath. On standing for thirty-six hours typical crystals of creatin separated. On filtering and evaporating to greater concentration a new but smaller crop of crystals was obtained.

The fluid, concentrated in this way, was treated with a moderate excess of 90 per cent. alcohol. The precipitate together with the crystals of creatin previously obtained was dissolved in water. On boiling this fluid with a trace of acid, creatinin was formed in abundance, as was shown by Weyl's reaction and by treatment with zinc chloride which gave typical crystals of creatinin-zinc chloride.

Hypoxanthin.—The alcoholic filtrate from the precipitated creatin was next evaporated nearly to dryness, a little water added the fluid made alkaline, filtered, and treated with an appropriate quantity of ammoniacal silver solution. The resultant heavy brown precipitate of nuclein bases on decomposition with hot nitric acid (sp. gr. 1.1) gave a yellowish filtrate which on cooling deposited a large proportion of crystalline substance consisting principally of typical needles of hypoxanthin silver nitrate. The mixture was allowed to stand for twenty-four hours for complete precipitation of this substance.

The filtrate from the crystals still contained nuclein base (xanthin) as was shown by the brown precipitate which appeared in small quantity when the fluid was again rendered alkaline and treated with ammoniacal silver solution.

The crystalline precipitate containing hypoxanthin silver nitrate was decomposed in a warm mixture of water and ammonium sulphide on the water-bath, the mixture filtered hot, the filtrate concentrated on a water-bath, saturated with ammonia and again filtered while hot. A comparatively large amount of hypoxanthin could be detected in this filtrate.

Guanin.—The substance insoluble in the ammoniacal fluid

yielded beautiful crystals of guanin. They were obtained by Horbaczewski's[24] method of treatment by solution in alkali and treatment with alcohol and acetic acid. The crystals were large, closely resembling those of creatinin zinc chloride.

The bulk of the crystalline extractives consisted of creatin, hypoxanthin, and guanin. We were unable to prove the presence of adenin and carnin though we occasionally obtained results by the qualitative methods which seemed to indicate the presence of these substances. No tests were made for other extractives.

It is interesting to note in this connection that guanin has been found in the ligaments of pigs with guanin gout.

The amount of crystalline extractives which we have found is too great to admit of the assumption that they were derived from the blood and lymph remaining in the tissue when the separation was begun. Normal blood contains only traces of nuclein bases and the tissue at the outset contained only traces of blood.

We are compelled to conclude that the creatin arises in the metabolic decomposition of tissue proteid, and that the nuclein base originates in the catabolism of nuclear proteid. Their presence in comparatively large amounts indicates the fairly high quantitative importance of these changes.

These results harmonize with those which we have obtained regarding the variety of proteids in the tissue, and emphasize our conclusion that the metabolism in this form of connective tissue may be of higher significance than has hitherto been supposed.

VIII. SUMMARY OF CONCLUSIONS.

1. Extracts of the ligamentum nuchae of the ox contain proteids which coagulate at 56°, 65°, 75°, and 82° C. Although these figures indicate identity with some of the albuminous substances of the blood, the coagulable proteids of ligament do not appear to have arisen wholly from lymph serum.

2. True nucleoproteid is present in the ligament to the extent of 0.056 per cent. of the fresh tissue. It contains at least 0.47 per cent. of phosphorus and on decomposition yields xanthin and phosphoric acid.

3. Ligament contains mucoid having the general qualities of

the other connective tissue glucoproteids. Analysis of five preparations gave the following average percentage results:

N	S	S as SO_3
13.44	1.61	1.06

4. By an improved method of preparation, which takes into account the presence of the other proteid constituents of the tissue, several samples of ligament elastin were made having the following average percentage composition:

C	H	N	S	O
54.12	7.33	16.87	0.15	21.53

All of these preparations contained sulphur. None of it could be split off as sulphide on boiling with caustic alkali.

A small proportion of the nitrogen of elastin may be split off in the form of hexone bases on decomposition with acid. The observation of Kossel and Kutscher that arginin and lysin are among the decomposition products was confirmed. Histidin was also discovered among the bodies separated in this way.

Elastin is not a "fat-proteid compound". No extractive material could be separated from our analyzed preparations by Nerking's process.

Purified elastin digests readily in pepsin-hydrochloric acid. Elastoses and true peptone are formed, protoelastose predominating in quantity. The amount of true peptone formed was surprisingly small even after long digestive periods, showing that elastoses are particularly resistant to progressive zymolysis.

The average combustion equivalent of four preparations of elastin was 5925 small calories.

5. The gelatin obtained from ligament has the following percentage composition:

C	H	N	S	O
50.49	6.81	17.90	0.57	24.23

These results indicate that the collagen of ligament is identical with that of bone and tendon.

The heat of combustion of ligament gelatin is equal to 5276 small calories.

6. Among the crystalline extractives obtainable from ligamentum nuchae were creatin, hypoxanthin and guanin.

In conclusion, it gives me great pleasure to acknowledge my indebtedness to Professor William J. Gies, at whose suggestion these experiments were begun, by whom much of the work was done, and to whose constant encouragement I owe its completion.

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